METHODS

FLUOROMETRIC TRIHYDROXYINDOLE METHOD OF DETERMINATION OF CATECHOLAMINES AND 3, 4-DIHYDROXYPHENYLALANINE IN URINE

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In the development of this method the writer made use of improvements in techniques introduced by various workers in order to permit, on the one hand, simplification of the method, and on the other hand, an increase in its sensitivity and specificity. These improvements include standardization of the concentration of eluate and buffers, so that the required pH of oxidation can be obtained instantly [3]; optimization of preparation of the lutin of noradrenalin (NA) in the presence of the corresponding concentration of alkali [2], stabilization of the fluorophores, and an increase in the intensity of their fluorescence by acidification to the corresponding pH [3, 4]; determination of adrenalin (A), NA, 3,4-dihydroxyphenylethylamine (DA), and 3,4-dihydroxyphenylalanine (D) in the same sample [1]. In addition, optimal conditions for simultaneous removal of catecholamines and D from the aluminum oxide, combination of the advantages of the column and tube methods of working with the adsorbent; recording fluorescence of DA after activation by heating of samples in which fluorescence of lutin of NA was measured; the ionic composition of the buffers enabling the intensity of fluorescence of the test substances to be increased; optimal pH, oxidation time, and concentration of NaOH and of alkaline ascorbates for A and D.

Reagents

All reagents were of the chemically pure and analytically pure grades, twice recrystallized in bidistilled water, metallic iodine was repeatedly sublimated under mild conditions, the percholoric acid was twice redistilled, and the ascorbic acid and Tris-buffer were recrystallized from aqueous solutions.

1. Brockman I neutral aluminum oxide was purified by boiling three times (10 min) with twice the volume of 1.5 N HCl; the sample was then decanted and repeatedly washed with bidistilled water until the reaction was weakly acid, after which it was activated by heating to 350°C for 6 h. 2. A 1 M solution of Tris-buffer. 3. 0.54 N and 0.03 N $HClO_4$ for elution. 4. EDTA-Na_2. 5. 2 N HCl to preserve the urine. 6. 2 N and 1 N solutions of NaOH made up from 1 N NaOH (high purity) after standing for many days. 7. 0.15M solution of Tris-buffer, pH 4.1 (about 8 ml 19 N formic acid was added to 1 liter 0.15 M solution of Tris-buffer), optimal for oxidation of A. 8. Tris-phosphate 0.085 M buffer solution, pH 7.25 (10.28 g Tris-buffer and 13.9 g $NaH_2PO_4.2H_2O$ were dissolved in water ; final volume 1 liter), optimal for oxidation of NA. 9. Tris-phosphate 0.85M buffer solution, pH 6.74 (made up from 0.085 N Tris-phosphate buffer solution, pH 7.25, to 1 liter of which was added 6.45 g $NaH_2PO_4.2H_2O$), optimal for oxidation of D. 10. A 0.25% solution of potassium ferricyanide (kept for not more than 2 weeks). 11. A 0.05 N solution of iodine (to 0.9 g Na was added 3 ml of a 0.4 N solution of iodine made up in 10% NaI, after which 21 ml water was added), kept for 1 month. 12. A solution of sodium ascorbate for determination of D (to 10 mg ascorbic acid dissolved in 0.25 ml water was added 4.75 ml 5 N NaOH and the mixture was thoroughly stirred), prepared immediately before use. 13. A solution of sodium ascorbate for determination of A (to 10 g ascorbic acid dissolved in 0.25 ml water 4.75 ml of 2.5 N NaOH was added and the mixture thoroughly stirred), made up immediately before use. 14. An alkaline solution of sodium sulfite (125 mg $Na_2SO_3.7H_2O$ and 44 mg EDTA were dissolved in 4.95 ml 2.02 N NaOH), optimal for obtaining lutin of NA and DA, prepared immediately before use. 15. A mixture of acids (glacial acetic acid, 6 N HCl mixed in the ratio of 1:1 by volume), to stabilize the lutins.

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0007-4888/80/8905-0701$07.50

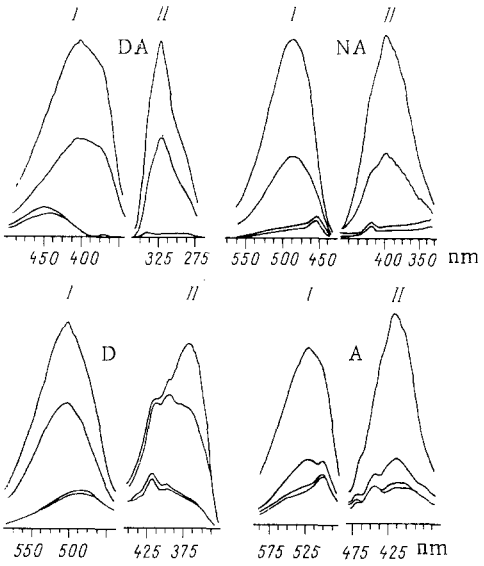

Fig. 1. Fluorescence spectra of DA, NA, D, and A. I) Emission spectra, II) excitation specta. Emission spectra recorded during excitation at: DA 325 nm; NA 390 nm; D 365 nm; A 425 nm. Excitation spectra recorded during recording emission at: DA 385 nm, NA 485 nm, D 485 nm, A 525 nm. Curves from bottom to top: tissue background during examination of native urine; tissue background after addition of corresponding amine to urine; fluorescence of amine present in native urine; fluorescence after addition of corresponding amine to this sample of urine. Both tissue backgrounds appear the same in the excitation spectrum of DA.

Equipment

Fluorescence was recorded on a fluorometer fitted with FEU-35 multiplier, SVD-120A illuminator, and a set of filters: adsorption — 313 nm, 365 nm, and 530 nm with halfwidth of transmission band of 30-40 nm; interference — 385 nm, 419 nm, and 481 nm with a halfwidth of transmission band of 17, 18, and 21 nm respectively. Chromatographic columns 10 mm in diameter with a glass filter (pores 40-50 μ), and with soft, airtight outlet stoppers.

Preparation of Urine

The urine was collected in bottles containing 2 N HCl (acting as a preservative), at the rate of 1 ml per 100 ml volume. The bottles were then kept at 0-4°C.

Method

Adsorption. Into a round-bottomed flask (50 ml) containing 60 mg EDTA 4 ml of filtered urine is added, and the mixture is shaken to dissolve the EDTA, and the pH adjusted to 8.5 ± 0.1 with 2 N NaOH solution. Next 500 mg Al_2O_3 is added to the flask, the contents are again shaken and the pH is measured, after which the flasks are gently shaken for 3 min so that the adsorbent is in a suspended state. The mixture is then applied to a column, particles of adsorbent settling in the flask are washed out with portions of the same urine escaping from the column. After all the urine has passed through the adsorbent the eluate is discarded and the column and adsorbent are carefully washed with water (once with 4 ml and then three times with 2 ml each time).

Elution

After application of 0.4 ml of 0.54 N $HClO_4$ to the column, the outlet of the column is hermetically closed and the column shaken five times in the course of 5 min, whereby

the advantages of the column and tube methods of elution can be combined. The outlet of the column is then opened and the eluate collected in beakers standing on ice. The adsorbent is washed successively with 0.6 ml of 0.03 N $HClO_4$ and 2.6 ml water. The total volume of eluate is 3.3 ml and its acidity 0.042 N $HClO_4$.

Formation of lutins and measurement of their fluorescence. The eluate is poured in volumes of 0.5 ml into tubes containing 0.6 ml of the corresponding buffer solutions, and as a result of mixing the optimal pH is created for oxidation of the substances (oxidation pH of A 3.8-4.0, of NA and DA 6.75 ± 0.15, and for D 6.4 ± 0.10).

Oxidation of A is carried out by the addition of 0.04 ml of 0.25% potassium ferricyanide. After shaking the tubes are allowed to stand for 3 min, after which 0.4 ml sodium ascorbate is added. Fluorometry is carried out immediately at 149-530 nm. The tissue background is formed on account of the fact that sodium ascorbate is added initially and potassium ferricyanide after an exposure of 3 min.

Oxidation of D and formation of the tissue background are carried out by means of the same reagents and in the same way as for A. The only difference is that to create the optimal pH for oxidation an appropriate buffer solution is used with sodium ascorbate; exposure for oxidation 2 min and fluorometry at 365-481 nm.

NA and DA are determined successively in the same tubes. Oxidation is carried out by the addition of 0.04 ml of a 0.5 N solution of iodine, with exposure of 3 min after shaking; 0.5 ml of alkaline sulfite is then added and after energetic shaking and exposure (5 min) 0.45 ml of a mixture of acetic and hydrochloric acids is added (final pH 4.2). In this case the tissue background is created by the acid medium, and for that reason a mixture of acetic and hydrochloric acids is added initially to the tube and not the buffer solution (as for A and D). The appropriate buffer solution is added after the alkaline sulfite. After the addition of all reagents (exposure 25 min) lutin of NA is examined fluorometrically at 365-481 nm. From the fluorometric cuvet the mixtures are again poured into tubes, where the DA fluorophore is activated for 15 min at 100°C. After removal from the thermostat the tubes are cooled to 20°C and the DA lutin examined fluorometrically at 313-382 nm.

The fluorometer is calibrated for quantitative determination of substances by addition of known substances to the urine, adjustment of pH to 8.5 immediately before addition of the adsorbent, followed by performance of all the operations of the method. Fluorescence of fluorophores of the added substances is calculated as the difference from the results of investigations of the same urine to which 0.01 N HCl solution was added instead of the known substance.

Elution of the substances is influenced by very many factors such as the rate and accuracy of alkalification of the urine, the purity of the reagents, their preparation at the right time, and the adsorptive activity of the aluminum oxide. Elution and oxidation pH of the amines require particularly careful verification. So that the necessary oxidiation pH for the monoamines could be obtained by mixing corresponding volumes of the eluate and buffers, the acidity of the eluate must be constant (0.042-0.40 N $HClO_4$). This is influenced by the following factors: 1) the degree of neutralization of the aluminum oxide during activation by heat (the more acid the adsorbent, the higher the acidity of the eluate); 2) the quantity of adsorbent; 3) the method of elution. During elution the $HClO_4$ is partially neutralized; the degree of neutralization depends on the method of elution, but with adequate contact between the eluting solution and the adsorbent, equilibrium arises. By the method of elution suggested in this paper, the 0.54 $HClO_4$ (0.4 ml) becomes 0.3 N after shaking. This concentration of $HClO_4$ enables 65-70% of the DA and 60-65% of the D to be removed from the adsorbent. The use of lower concentrations of acid for elution sharply reduces the outflow of D and DA (A and NA are eluted well by lower concentrations also). Combining the tube and column methods of elutions, on the one hand, and the small volume of the eluting solution, on the other hand, enable a stable concentration on 0.3 N $HClO_4$ above the adsorbent to be created at the moment of elution and ensure optimal concentration (0.042 N) in the end volume of the eluate.

After preparation of a new portion of adsorbent, control titration of the eluate or measurement of the pH of the mixture of corresponding volumes of eluate and buffers must be carried out. If the acidity of the eluate is high, either repeated activation of the adsorbent or reduction of the concentration of the eluting solution is essential.

LITERATURE CITED

1. É. Sh. Matlina, Z. M. Kiseleva, and É. I. Sofieva, in: Methods of Investigation of Certain Hormones and Mediators [in Russian], Moscow (1965), p. 25.
2. D. Loverty and K. M. Taylor, Anal. Biochem., 22, 269 (1968).
3. J. F. O'Hanlon, Jr., H. C. Campuzano, and S. M. Horvath, Anal. Biochem., 34, 568 (1970).
4. A. S. Welch and B. L. Welch, Anal. Biochem., 30, 161 (1969).